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Comparative analysis of pistil transcriptomes reveals conserved and novel genes expressed in dry, wet and semi-dry stigmas

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Abstract

Fertilization in angiosperms depends on a complex cellular 'courtship' between haploid pollen and diploid pistil. These pollen-pistil interactions are regulated by a diversity of molecules, many of which remain to be identified and characterized. Thus it is unclear to what extent these processes are conserved among angiosperms, a fact confounded by limited sampling across taxa. Here we report analysis of pistil-expressed genes in *Senecio squalidus* (Asteraceae), a species from euasterid II, a major clade for which there is currently no data on pistil-expressed genes. Species from the Asteraceae typically have a 'semi-dry stigma', intermediate between the 'wet' and 'dry' stigmas typical of the majority of angiosperms. Construction of pistil-enriched cDNA libraries for *S. squalidus* allowed us to address two hypotheses: i) stigmas of *S. squalidus* will express genes common to wet and dry stigmas and genes specific to the semi-dry stigma characteristic of the Asteraceae, and ii) genes potentially essential for pistil function will be conserved between diverse angiosperm groups and therefore common to all currently available pistil transcriptome data sets, including *Senecio*. Our data support both these hypotheses: The *S. squalidus* pistil transcriptome contains novel genes and genes previously identified in pistils of species with dry stigmas and wet stigmas. Comparative analysis of the five pistil transcriptomes currently available (*Oryza sativa, Crocus sativus*, *Arabidopsis thaliana, Nicotiana tabacum*, and *Senecio squalidus*), representing four major angiosperm clades and the three stigma states, identified novel genes and conserved genes potentially regulating pollen-pistil interaction pathways common to monocots and eudicots.

(247 words)

Introduction

Rapid and reliable communication between the male (pollen) and female (pistil) reproductive tissues is essential for successful fertilisation in angiosperms. The tissue of the pistil acts as a physical and chemical interface between the male and female gametophytes, beginning at the stigma surface during pollen germination and continuing until successful fertilisation at the ovule (Hiscock and Allen, 2008). Many different processes occur simultaneously in pistil tissues, including species recognition, self-incompatibility (SI), pollen hydration, pollen tube growth and pathogen defence (see Swanson et al., 2004; Malho et al., 2006; Wilsen and Hepler, 2007; Hiscock and Allen, 2008, for reviews). It is clear that a high diversity of both pollen and pistil molecules mediates the complex interactions between these two tissues, but, with the exception of SI, relatively little is known about the molecular pathways involved (Swanson et al., 2004; Sanchez et al., 2004; Edlund et al., 2004). In addition to the large range of molecules present in the pistil tissues of a given species, it is likely that a number of specific molecules have diversified between taxa (Swanson et al., 2004). There are two reasons for this: firstly, genes that are involved in regulating sexual reproduction are likely to evolve at a higher rate than those controlling housekeeping processes (Swanson and Vacquier, 2002), and secondly genes involved in maintaining species boundaries will be, by their nature species-specific, and hence highly diverse between species (Swanson et al., 2004; Edlund et al., 2004). To date much research into pollen-pistil interactions has focussed on SI and these studies have shown that a high diversity of molecules and processes are employed for the same role of recognizing and rejecting self-pollen (for reviews see Hiscock and McInnis, 2003; Takayama and Isogai, 2005; Franklin-Tong, 2008). This suggests that a similar or greater diversity of molecules is likely to regulate compatible pollen-pistil interactions. Certainly, despite the identification of an increasing number of molecules implicated in compatible pollen-pistil interactions, there are few examples of shared genes between species (Lord 2003, Hiscock and Allen, 2008). However, these types of studies have focussed on specific genes in model species, and the low consensus so far observed between species is largely a consequence of this limited sampling. It is therefore important to extend studies of genes and proteins regulating pollen-pistil interactions to include other species from diverse families of flowering plants. One approach to identifying more genes involved in pollen-pistil interactions in diverse angiosperm species is through the

generation of pistil-enriched cDNA libraries, which allows comparison of tissue-specific transcriptomes between species.

Recently, several studies have identified genes expressed in the reproductive tissues of *Arabidopsis thaliana*, *Oryza sativa* (rice), *Crocus sativus* (crocus), and *Nicotiana tabacum* (tobacco), representing three major angiosperm clades – monocots (rice and crocus), rosids (*Arabidopsis*) and asterid I (tobacco) (APG II, 2003). Large data sets have been generated to represent the transcriptomes of pollen and/or pistil tissues at different stages of development (Swanson et al., 2005; Tung et al., 2005; Becker et al., 2003; Li et al., 2007; D'Agostino et al., 2007; Quiapim et al., 2009).

Angiosperm stigmas can be broadly classified as either 'wet' or 'dry', depending on the presence or absence of secretions at the stigma surface (Heslop-Harrison and Shivanna, 1977). Taxa possessing wet stigmas typically secrete an exudate, which allows pollen hydration and germination to occur, even with pollen from other species. By contrast, in species with dry stigmas, pollen adhesion and recognition often precede hydration, and these processes are highly regulated (Dickinson, 1995). *A. thaliana, O. sativa* and *C. sativus* possess dry stigmas, whereas *N. tabacum* has a wet stigma (Heslop-Harrison and Shivanna, 1977, Fig. S1).

To expand phylogenetic sampling of pistil-enriched/pistil-specific transcriptome data sets to include the asterid II clade we have used suppression subtractive hybridization to construct pistil-enriched cDNA libraries for *Senecio squalidus* (Oxford Ragwort, Asteraceae). Importantly, *S. squalidus* possesses a 'semi-dry' stigma that shows features intermediate between dry and wet stigmas (Hiscock et al., 2002). Like species with dry stigmas the *S. squalidus* stigmatic papillae possess a surface cuticle, but unlike the typical dry stigma (e.g *Brassica sp*.) this is not continuous, and does not extend to the base of the papillae, and when stigmas reach maturity a small amount of extracellular secretion is present in these basal regions between papillae (Hiscock et al., 2002). The semi-dry stigma state, which appears to be common to all members of the Asteraceae (Hiscock et al, 2002; Allen and Hiscock, 2008), the second largest family of flowering plants, has not been well studied at a molecular level and its evolutionary relationship with wet and dry stigmas is unknown.

The availability of five pistil transcriptome data sets from four major angiosperm clades that together contain >90% of flowering plants has allowed us to compare the diversity and conservation of pistil-expressed genes in species with wet, dry, and semi-dry stigmas across the monocots (*C. sativus,* Liliales and *O. sativa*, Poales) and

eudicots (*A. thaliana*, Brassicales*, N. tabacum,* Solanales and *S. squalidus,* Asterales) [Fig. S1]. Using these data we investigated two hypotheses: i) the 'semi-dry' stigma of *S. squalidus* will express genes common to species with both wet and dry stigmas, as well as expressing genes specific to the semi-dry stigma of the Asteraceae, and ii) specific classes of genes potentially essential for pistil function will be conserved between diverse angiosperm groups and therefore common to the five pistil transcriptomes sampled.

Results

Identification and functional classification of putative pistil-specific genes

Three cDNA libraries were created from the cloned products of suppression subtractive hybridisation, each corresponding to pistil-expressed sequences from three different *S*-genotypes (S_1S_2, S_1S_3, S_1S_4) of *S. squalidus*. Approximately 80 cDNA clones from each library were selected on the basis of the differential screening results (hybridisation to subtracted pistil probe versus no hybridization to leaf probe) and sequenced. Functional annotation of the putative pistil-specific genes was performed using the BLAST-X algorithm. To generate a representative dataset of the *S. squalidus* transcriptome, the three subtracted libraries were combined and assigned functional categories (Table I). A total of 174 cDNA clones was identified and of these 86% (150 cDNA clones) could be assigned a putative function according to the Gene Ontology database. The 174 cDNA clones corresponded to 115 different genes, with several genes present in multiple copies in the cDNA libraries. Over 50% of the genes identified were assigned functions in just four functional categories: metabolism (22%); transport (15%); signalling (11%); cell wall related (10%) (Fig. 1).

Confirmation of pistil-specific expression by northern hybridisation.

To confirm the expression of candidate genes in pistil tissues, northern hybridisation was performed on a subset of genes in the dataset (Fig. 2). All six cDNA clones were expressed in pistil tissue, but not in leaf tissue, indicating that the suppression subtraction had worked efficiently. Four of the clones (encoding a nodulin protein, a membrane-associated protein, a myo-inositol oxygenase protein and a nematode resistance protein) were expressed exclusively in the pistil (Fig 2C-F), and two (encoding a cytochrome p450 and a calcium binding kinase) were also expressed in

pollen (Fig 2A-B). The nematode resistance gene is expressed as several different sized transcripts in the pistil.

Comparison of the *Senecio squalidus, Arabidopsis thaliana, Oryza sativa, Nicotiana tabacum* and *Crocus sativus* pistil transcriptomes

Pistil-specific datasets have so far been generated in just 2 species: *Arabidopsis thaliana* (Tung et al., 2005; Swanson et al., 2005) and *Oryza sativa* (Li et al., 2007). The *A. thaliana* datasets were generated from microarray analysis and cDNA subtraction of stigma tissue (Swanson et al., 2005) and microarray analysis of stigma/style tissue (Tung et al. 2005). The *O. sativa* dataset was produced from analysis of an Affymetrix rice whole-genome array and cDNA microarray comparisons of stigma tissue (Li et al., 2007). Additional data for pistil transcriptomes has also been generated recently in *Crocus sativus* (D'Agostino et al., 2007) and *Nicotiana tabacum* (Quiapim et al., 2009). The *N. tabacum* pistil EST dataset was generated from stigma and style cDNA (Quiapim et al., 2009), and the *C. sativus* EST dataset from stigma cDNA (D'Agostino et al., 2007). Comparisons of the results generated by these separate studies with our data set from *S. squalidus* showed broad correlations in the functional gene classes identified (Fig. 1, Table II). General classification of the stigma-enriched datasets of *S. squalidus, A. thaliana* and *O. sativa* revealed that the proportions of genes in each functional class were similar. The proportions of genes in the functional classes: transcription, cell wall, stress/defence, signal transduction and unclassified from *S. squalidus* were comparable to those of *A. thaliana* and *O. sativa*, but *S. squalidus* had the highest proportions of genes involved in transport and metabolism. A Wilcoxon-signed rank test on the proportion data confirmed that the results were not significantly different from each other (*S. squalidus/A. thaliana,* $P = 1.00$; *S. squalidus/O. sativa,* $P = 0.484$; *A. thaliana/O.sativa,* $P = 0.674$).

Two independent studies in *A. thaliana* have indicated that the categories of metabolism, stress/defence, signalling and cell wall-related contain a large proportion of pistil-specific genes (Tung et al., 2005; Swanson et al., 2005). A similar study in *O. sativa* identified the categories of cell-wall related, stress/defence and signal transduction as being the largest categories, and also reported a large number of genes involved in transcription (Li et al., 2007). Correlations between the *S. squalidus, A.*

thaliana and *O. sativa* data sets have highlighted certain functional groups that contain high numbers of pistil-specific genes in all three species. In particular these were cell-wall related and signalling, with the categories of transport, stress/defence and metabolism also containing a high percentage of pistil-specific genes.

Analysis of conserved pistil-specific genes

In addition to the similarities in the patterns of functional annotation of the pistil-enriched datasets, a number of pistil-enriched gene classes were common to three or more different species (Table II). Gene families that were consistently detected in the pistil tissues included cytochrome p450, ABC transporters and lipid transfer proteins. A greater number of shared genes were identified between *S. squalidus* and the dry stigma species (*A. thaliana. O. sativa* and *C. sativus*) than with the wet stigma species (*N. tabacum*). This may be a consequence of the smaller *N. tabacum* pistil preferential dataset or may reflect fundamental differences between wet and dry stigmas. Sequence analysis of three conserved proteins indicated structural and functional similarity (Fig. 3). Lipid-transfer proteins identified in the pistil-enriched datasets of *Arabidopsis* (At2g38530, At2g38540, At5g59310, At5g01870), rice (AK105838), *Crocus* (EX146511, EX1483990) and *Senecio* showed sequence homology to SCA (Q9SW93; ~50% protein identity), a LTP identified in *Lilium longiflorum* (Park and Lord, 2003) and shown to function in pollen tube adhesion and guidance (Chae et al., 2007) (Table II, Fig. 3a). All twenty feature residues of the hydrophobic cavity characteristic of this gene family were conserved in the *Senecio* sequence. Additionally there were eight cysteine residues conserved between the sequences from all species (Fig. 3a). A further cysteine-rich protein of unknown function was also identified in three out of five of the study species; *Senecio*, *Arabidopsis* (At2g37110) and rice (NP_001042073) (Table II) with a high level of sequence conservation (60% identity between rice and *Arabidopsis*, 71% identity between *Arabidopsis* and *Senecio*) (Fig. 3b).

A pistil-specific gene showing sequence similarity to a member of the nodulin/mtn3 gene family was identified in separate studies of *Arabidopsis* (At1g21460, At5g53190), rice (CAE04315), tobacco (TOBC023B06) and *Senecio.* Seventeen copies of this gene have been identified in *Arabidopsis* (Guan et al., 2008) and eighteen in rice genomes (Chu et al., 2006; Yang et al., 2006). Two of the *A. thaliana*

orthologues were shown to be pistil-specific (At1g21460 and At5g51390) and a further two pollen-specific (At5g62850 and At5g40260). Interestingly, the *S. squalidus* protein exhibits higher homology to the *N. tabacum* (C023B06, C061G07) and *O. sativa* (CAE04315) proteins than any of the *Arabidopsis* proteins. All the nodulin/mtn3-like protein sequences share 7 transmembrane regions and 2 conserved intracellular regions, but have more variable extracellular regions (Fig. 4c). Further analysis of this protein family in *Arabidopsis* indicated a complex pattern of evolution of pistil- and pollen-specific proteins, with the *Senecio* and rice pistil-specific proteins allying to different *Arabidopsis* clades (Fig. 4). The *Senecio* pistil-specific nodulin shares greater sequence identity with the *N. tabacum* sequence (55 %) than those from *Arabidopsis* and rice (35 %, 31 %, respectively).

Novel pistil-specific genes in *S. squalidus*

The pistil cDNA libraries in *Senecio* contain a number of novel genes not previously identified as pistil-specific in other species and not present in our *Senecio* floral database (www.seneciodb.org). These include a WNK (With No K/lysine) kinase with a putative calcium-binding domain, a membrane-associated protein, a nematode resistance protein, and several hypothetical proteins of unknown function (Table I). The identification of novel pistil-specific genes in *S. squalidus* is particularly interesting, as this species possesses a sporophytic SI (SSI) system, which operates through a different mechanism to the well-characterised SSI system found in the Brassicaceae (Hiscock et al., 2003; Tabah et al., 2004). The *Senecio* pistil dataset is therefore expected to contain genes potentially involved in mediating the female side of SSI, including primary *S*-recognition genes.

Discussion

The *S. squalidus* pistil-enriched transcriptome

Suppression subtractive hybridisation was used successfully to isolate pistil-enriched transcripts from cDNA libraries constructed for three different *S*-genotypes of *S. squalidus*. When combined, these yielded 115 different candidates for pistil-specific genes for *S. squalidus*. Differential screening of the cDNA libraries confirmed the expression of clones in pistil tissue**.** Northern blot analysis, performed on a subset of cDNA clones, revealed that expression of the majority of these genes was exclusive to pistil tissue. Both these methods of screening illustrated the efficiency of SSH for

isolating tissue-specific genes. Pistil-specific genes are likely to play important roles in many stigma functions, including: defence, pollen adhesion and hydration, pollen tube guidance and structural support within the ECM, and SI. The pistil-enriched libraries created for *S. squalidus* have identified potential components of all these systems, and may be used to compliment and confirm information from other species for which pistil transcriptome data is available. We therefore compared all five available pistil transcriptomes - *Oryza sativa* and *Crocus sativus* (monocots), *Arabidopsis thaliana* (rosid clade), *Nicotiana tabacum* (euasterid I clade), and *Senecio squalidus* (euasterid II clade) spanning four major angiosperm clades that contain >90% of all flowering plants.

All five pistil transcriptomes show broad similarities in the proportion of genes in particular functional classes, the types of genes identified, and sequence similarities between protein products. Additionally, the *S. squalidus* SSH libraries contained a number of genes showing orthology to pistil-specific genes previously identified in other species (Park and Lord, 2003; Otsu et al., 2004; McInnis et al., 2005). Comparisons with data from other species and extensive *Senecio* EST databases (www.seneciodb.org) also identified a number of novel genes in the *Senecio* pistil cDNA libraries (Table I). These are likely to correspond to rare transcripts in the pistil transcriptome and highlight the usefulness of SSH as a technique that is able to identify genes expressed specifically in the tissue of interest.

Comparisons of the currently available pistil datasets highlighted differences between results from whole-pistil and pistil-specific studies. The mature stigma dataset from *C. sativus* contained a large number of genes involved in metabolism, transport and transcription (D'Agostino et al., 2007, 157 genes). The over-representation of metabolism genes in *C. sativus* compared to the other dry stigma species may be a consequence of the data set being a sample of the unsubtracted pistil transcriptome, not just pistil-specific transcripts. Alternatively, these differences may reflect the high levels of carotenoid metabolism in the highly specialised stigma of *C. sativus* (D'Agostino et al., 2007). A comparison of the functional categories of the *A. thaliana* pistil dataset at different resolutions of pistil specificity revealed differences in proportions of functional classes, highlighting classes that were important in general cellular function (plastid-related genes) and those required for pistil-specific

processes (cell wall, ER, response to stress, plasma membrane) (Hiscock and Allen, 2008).

Comparative analyses of wet, dry and semi-dry stigma/style transcriptomes

When the data generated by the present study were compared with data from *Arabidopsis thaliana, Nicotiana tabacum, Oryza sativa* and *Crocus sativus*, similarities and differences were revealed (Swanson et al. 2005; Tung et al. 2005; Quiapim et al., 2009; Li et al., 2007; D'Agostino et al., 2007). The study species differ in relatedness, pistil structure and function, and in specific pollen-pistil processes, allowing the opportunity to assess consensus across diverse taxa (Fig. S1). Differences were observed between the dry stigma species (*A. thaliana, O. sativa, C. sativus*) and wet stigma species (*N. tabacum*) datasets, most notably in the proportions of genes in each functional class, and in the types of genes expressed exclusively in the pistil (Table II). The largest functional categories in the *N. tabacum* pistil transcriptome were post-translational modification/protein turn-over, translation and energy production and conversion, suggesting that the *N. tabacum* pistil is composed of highly active metabolic cells, perhaps reflecting the active production of wet surface secretion, a defining feature of wet stigma species (Quiapim et al., 2009).

Data from dry stigma species consistently highlighted categories of cell wall-related and signalling as containing large numbers of genes and being over-represented in the dry stigma (Swanson et al., 2005; Tung et al., 2005; Li et al., 2007; Hiscock and Allen, 2008). A detailed comparison of the *A. thaliana* and *N. tabacum* datasets revealed a low percentage of homologous sequences between the two species (Quiapim et al., 2009). In contrast, comparisons of the rice and *Arabidopsis* datasets revealed 83 similar sequences, most of which belonged to cell wall-related and signal transduction groups, indicating conservation of these functions in the dry stigma. The *S. squalidus* dataset shared a greater number of homologous genes with the dry stigma species (*A. thaliana,* 37; *O. sativa,* 46; *C. sativus*, 39) than the wet stigma species (*N. tabacum,* 8); although a more thorough comparison involving additional species would be needed to confirm these results. However, similarities were also detected between the tobacco and *Senecio* pistil datasets; most notably a high number of extensin-like gene transcripts, a class of gene that has been well studied in the tobacco pistil (de Graaf et al., 2003). It is interesting to observe similarities between *S.*

squalidus and species with wet stigmas and dry stigmas because the semi-dry stigma of *S. squalidus* (Hiscock et al., 2002) may represent a derived form of either.

Evidence of conserved pistil-specific genes in diverse angiosperm groups

Despite the differences in gross pistil morphology between these diverse species, certain features of the pollination process appear to be shared: in particular the presence of lipids at the pollen-stigma interface, the involvement of small cysteine-rich proteins in pollen-stigma interactions and the role of water as a directional cue for the pollen tube (Hiscock and Allen, 2008). The present study was able to identify additional potential correlations between the pistil datasets and individual pistil-specific genes, with several genes appearing to be restricted to wet, dry or semi-dry stigma transcriptomes (detailed below). In particular the protein sequences of three genes (a lipid transfer protein, a cysteine-rich protein and a nodulin/mtn3 protein) from different species were aligned and exhibited sequence similarity and conservation of functional residues (Fig. 3). These examples of conserved pistil-specific genes may represent ancient pistil processes that have been maintained in diverse angiosperm species across the monocot-eudicot divide. Alternatively these genes may be evidence of the convergent evolution of classes of genes to acquire pistil-specific functions. There is a clear need for studies of the pistil transcriptomes of lower eudicots and basal angiosperms to answer these critical evolutionary questions.

Cysteine-rich proteins

Our comparative transcriptome analysis provides further support for the hypothesis that cysteine-rich proteins play important and varied roles during the pollen-pistil interaction (Verhoeven et al., 2005; Chae et al., 2007; Tang et al., 2004; Takayama et al., 2000; Doughty et al., 1998). Several Cys-rich proteins were identified in the pistil-enriched data sets of *Senecio*, *Arabidopsis*, rice, tobacco and *Crocus*. Two of these, a cys-rich protein of unknown function and a lipid transfer protein, were aligned to demonstrate sequence similarity and conservation of functional residues between the different species, suggesting a potential conserved role of these proteins in pistil function (Fig. 3). The cys-rich protein of unknown function exhibited particularly high sequence similarity between the dry/semi-dry stigma species *S. squalidus, A. thaliana* and *O. sativa* (60-70% identity). This protein was not detected in the

tobacco pistil transcriptome, suggesting a role specific to dry/semi-dry stigmas only. The lipid transfer protein was detected in all species studied, and typically was expressed at high levels in the pistil (Tung et al., 2005; Swanson et al., 2005; Quiapim et al., 2009). The tobacco sequence exhibits very high sequence identity (83.4%) to LTP (Q03461), a protein that has been shown to mediate cell-wall loosening activity in the stigma exudate (Nieuwland et al., 2005). These LTP's are also related to another LTP, SCA (Q9SW93), which is involved in pollen tube adhesion and guidance in lily (Park and Lord, 2003; Kim et al., 2006; Chae et al., 2007).

Cell-wall-related proteins

Within the cell-wall-related functional category several classes of genes were present in data sets from all five study species. These included extensin-like proteins and hydroxyproline-rich glycoproteins, consistent with previous work that has shown these classes of protein to be ubiquitous components of transmitting tissue of the pistil through which pollen tubes grow and navigate to the ovules (Wu et al., 2001). Both the *S. squalidus* and *N. tabacum* datasets contained large numbers of extensin-like genes, suggesting that this class of gene is particularly important in wet and semi-dry stigmas, in contrast to the *Arabidopsis,* rice and *Crocus* datasets which each contained just one extensin-like gene. In *N. tabacum* these proteins have been implicated to function in a range of different processes within the pistil including pollen-tube guidance and SI (de Graaf et al., 2003; Cheung et al., 1993; Wu et al., 1995; Hancock et al., 2005). Another putative pistil-specific cell-wall component identified in *S. squalidus* was a pectinesterase, a class of enzymes also present in the data sets of rice, *Arabidopsis* and *Crocus,* highlighting their importance in dry and semi-dry stigma function. Pectinesterase-like proteins are hypothesised to function in enabling pollen tube growth through the papilla cell wall by mediating cell wall loosening and expansion (Bosch et al., 2005; Bosch and Hepler, 2006).

Signalling genes

In the signalling class of pistil-specific proteins, a relatively large number of receptor-like protein kinases are present in the *S. squalidus* data set, consistent with results from other species (Swanson et al., 2005; Tung et al., 2005; Quiapim et al., 2009; Li et al., 2007; D'Agostino et al., 2007). It is likely that these pistil-specific kinases are specialised to co-ordinate pistil development, defence responses and to facilitate

communication between the pollen and pistil, with downstream implications on pollen tube growth (Johnson and Preuss, 2003). This class of proteins have been studied in both dry and wet stigmas of the Brassicaceae and Solanaceae, respectively. The most extensively-studied example of a stigma-specific receptor kinase is the *S*-receptor kinase (SRK) from the Brassicaceae, which regulates the female response in SSI (Takayama and Isogai, 2005). Pollen-specific receptor-like kinases (Muschietti et al., 1998) have been shown to interact with corresponding pistil cysteine-rich protein ligands LAT52 (Tang et al., 2002) and LeSTig1 (Tang et al. 2004) and are thought to mediate pistil response to pollen tube growth in tomato.

Transport genes

The *Senecio*, *Arabidopsis* and rice pistil-enriched data sets contain a significant proportion of genes that potentially play a role in transport within the pistil, suggesting that this function is particularly important in dry and semi-dry stigmas. All five species datasets contained at least one member of the ABC transporter family, a family of proteins that function in the transport of a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids and sterols (Sidler et al., 1998). The *Senecio* and *Arabidopsis* (NP_181467) sequences exhibited high sequence similarity (74% and 73% identity respectively) with a pistil-specific ABC transporter gene, NtWBC1 (*Nicotiana tabacum* ABC transporter of the white-brown complex subfamily, AAR06252), which has been characterized in tobacco (Otsu et al., 2004). Expression of NtWBC1 is localised to the stigmatic secretory zone, to the cells that produce the stigmatic exudate and it is thought that this protein may be involved in the transfer of lipids to the stigmatic exudate (Otsu et al., 2004). It may be hypothesised that the orthologue of NtWBC1 acts in a similar way in the semi-dry stigma of *S. squalidus,* where secretion of the surface exudate is enhanced in stigmatic papillae after pollination (Hiscock et al., 2002).

Another common feature of pistil function is the presence of a stigmatic water gradient acting as an initial directional cue for pollen tube growth (Hiscock and Allen, 2008). In dry stigma species, the presence of water at the stigma surface is highly regulated (Dickinson, 1995). In *Brassica*, a stigma-specific aquaporin (MIP-MOD, AAB61378) has been implicated in the regulation of water flow into the pollen grain during grain hydration (Dixit et al., 2001). The pistil-specific aquaporins identified in

Senecio, Arabidopsis and rice may therefore have a similar role. In support of this suggestion, the *Senecio* pistil-specific aquaporin shares 85% sequence identity with the *Brassica* MIP-MOD protein. This putative role of aquaporins in pollen hydration may therefore explain why they were not detected in the tobacco pistil preferential dataset, since the presence of the secreted stigmatic exudate in tobacco precludes the need for control of water flow to the stigma surface (Wolters-Arts et al., 2002).

Stress/defence genes

Our comparative transcriptome analyses have identified a number of genes encoding proteins potentially involved in stress/defence responses. This is particularly interesting as there is evidence that the molecules involved in pollination and stress/defence responses may be related evolutionally and functionally (Vogt et al., 1994; Li et al., 2007). Indeed, many authors have proposed that certain SI mechanisms may have arisen through modification of pre-existing pathogen defence mechanisms (de Nettancourt, 1977; Hodgkin et al., 1988; Elleman and Dickinson, 1999; Dickinson, 1995). Several of the genes involved in stress/defence responses identified in the *Senecio* pistil data set may be involved in reactive oxygen species (ROS) signalling. Stigmatic tissues have been shown to accumulate high levels of ROS constitutively, particularly hydrogen peroxide (H_2O_2), and it has been hypothesised that the high levels of ROS/H_2O_2 may protect stigmas against pathogen attack (McInnis et al., 2006a). ROS respond to external stimuli, acting as early messengers in signalling cascades by inducing the expression of a number of genes including pathogenesis-related (PR) protein genes (Sepúlveda-Jiménez et al., 2005). For instance in the leaves of *Beta vulgaris*, a UDP-glucosyltransferase is induced by high levels of ROS, which accumulate as a response to wounding and bacterial infection (Sepúlveda-Jiménez et al., 2005). The *Senecio* pistil-specific UDP-glucosyltransferase may therefore respond in a similar way to the high levels of ROS in the stigmatic tissue, and act downstream by glucosylating hormones and secondary metabolites. UDP-glucosyltransferases were detected in the pistil datasets of all the dry stigma species, suggesting conservation of their function in pistils.

Another gene identified in the *Senecio* pistil data set showed high sequence similarity (71% protein identity) to an extracellular dermal glycoprotein (EDGP, BAA03413) from carrot (Shang et al., 2005) and the Nectarin IV protein (67% sequence identity,

AAX81588) from tobacco (Saqlan Navqi et al., 2005). Both EDGP and Nectarin IV belong to a newly-identified superfamily of inhibitor proteins (Saqlan Navqi et al., 2005). Nectarin IV is expressed in the nectary of ornamental tobacco plants during anthesis until after fertilization, when expression peaks (Saqlan Navqi et al., 2005). Analogies have been made between the high levels of ROS and H_2O_2 detected in the stigma, and the equally high levels in nectar, which are hypothesised to protect against pathogen attack (Carter and Thornburg, 2004; McInnis et al., 2006b). Recent studies in *Senecio* have shown that levels of ROS/H_2O_2 were reduced in stigmatic papillae to which pollen grains had adhered suggesting that NO from pollen may be acting to reducing ROS/H_2O_2 abundance in stigmatic papillae, potentially to allow pollen to be distinguished from fungal pathogens. The presence of catalase transcripts in the *Senecio* pistil libraries indicates that the pistil cells are capable of actively breaking down H_2O_2.

In addition to the putative EDGP/Nectarin IV orthologue, a gene showing sequence similarity (59% identity) to Nectarin 1 from *Petunia* (NEC1, AAG34696) was also identified in the *Senecio* pistil-enriched data sets. NEC1 belongs to the mtn3/saliva family of proteins (Ge et al., 2000). A homologue of another member of this family, nodulin/mtn3, was also identified in the *Senecio, Arabidopsis*, tobacco and rice pistils. Members of this protein family have been implicated in diverse cellular processes, including disease resistance and pollen development, although their specific function is unknown (Guan et al., 2008; Yang et al., 2006). In the *Arabidopsis* and rice genomes, nodulin/mtn3 proteins are present in high copy number (17 and 18 genes, respectively). In *Arabidopsis,* at least four members of this gene family are expressed exclusively in reproductive tissues (Fig. 4). Identification of pistil-specific proteins in four of the pistil preferential datasets (Table II; Fig. 3) highlighted the potential importance of these genes in reproductive tissues, where their function may be conserved across diverse taxa. Sequence analysis showed the *Senecio* protein to lie within a sub-clade of this family, which also contained NEC1 and Xa13/Os8N3 (ABD78942), a disease-resistance gene for bacterial blight of rice (Fig. 4; Chu et al., 2006).

Conclusion

The aim of this study was to expand upon currently available data for genes expressed in pistils by analysing the pistil transcriptome of a species from the Asteraceae (*Senecio squalidus*). *S. squalidus* possesses a 'semi-dry' stigma, intermediate between the 'wet' and 'dry' stigmas typical of most angiosperms and all species previously analysed at the level of pistil gene expression. By selecting a species from the Asteraceae for analysis we were also able to sample a representative of the asterid II clade, a major angiosperm clade for which there was no data on pistil-expressed genes prior to our study. We were therefore able to explore two key hypotheses: i) that the 'semi-dry' stigma of *S. squalidus* will express genes common to species with wet and dry stigmas, as well as expressing genes specific to the semi-dry stigma of the Asteraceae, and ii) that certain classes of genes potentially essential for pistil function will be conserved between diverse angiosperm groups and therefore common to the pistil transcriptomes of the five angiosperm species sampled to date. Overall, our findings support both these hypotheses. With respect to hypothesis i) our data show that the pistil transcriptome of *S. squalidus* is generally more similar to the dry stigma transcriptome of *Arabidopsis* but also contains genes expressed in wet stigmas. *S. squalidus* pistils also express a number of unique genes that could potentially be involved in the novel mechanism of sporophytic SI found in the Asteraceae.

With respect to hypothesis ii) our comparison of the five available pistil transcriptomes - *Oryza sativa* (rice) and *Crocus sativus* (crocus), representing monocots, and *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), and *S. squalidus,* representing three divergent clades of eudicots, identified genes encoding a number of potentially orthologous proteins, most notably a lipid transfer protein, a cysteine-rich protein, and a nodulin/mtn3 protein. It is likely that these proteins play similar roles in their respective species, and may represent components of the pollen-pistil interaction machinery system common to the majority of the angiosperms. This suggests that some of the complex interactions underlying pistil function in diverse species with wet, dry and semi-dry stigmas are shared and have been inherited from the common ancestor of monocots and eudicots (Hiscock and Allen, 2008).

Materials and methods

Plant material

All *Senecio squalidus* plants were grown in glasshouse conditions, according to Hiscock (2000). *S*-genotyped individuals (S_1S_2, S_1S_3 and S_1S_4, Brennan, Tabah, Harris, and Hiscock, submitted) were used for RNA and DNA extraction.

Suppression subtractive hybridisation

Total RNA was extracted from leaf and pistil tissue from plants of three different *S*-genotypes (S_1S_2, S_1S_3 and S_1S_4) using the TRIzol® reagent, according to the manufacturer's instructions (Invitrogen). cDNA was then synthesised from total pistil and leaf RNA using the SMART PCR cDNA Synthesis kit (Clontech). Subtraction was performed separately for each genotype using cDNA from pistil and leaf tissue using the PCR-Select cDNA subtraction kit (Clontech). This technique includes a normalization step to equalize the abundance of transcripts, allowing comparisons of copy number to be made. Reverse subtractions were also performed for each genotype for the purposes of differential screening. The subtracted PCR products were cloned using the TOPO TA-cloning kit (Invitrogen) and screened for inserts. All colonies containing inserts were picked and transferred to separate wells of a 96-well plate containing Luria-Bertani (LB) broth.

Colony arrays were created by dot-blotting cultures of each clone onto a nylon membrane (Hybond-NX, GE Healthcare), placed on the surface of a plate of LB agar containing 100 μg/ml ampicillin and incubated at 37˚C overnight. The membrane was denatured, neutralized, and DNA fixed to the membrane by baking for 1.5 hours at 80˚C. The resulting subtracted libraries were differentially screened using total leaf cDNA, total pistil cDNA, subtracted leaf cDNA and subtracted pistil cDNA as probes. Probes were prepared using Ready-To-Go™ DNA Labelling Beads (GE Healthcare) and labelled with [α^{-32P}] dCTP (Amersham Biosciences). Hybridisation was performed in Southern hybridisation solution (300mM $NaPO_4$ buffer, 7% SDS, 1mM EDTA, 10mg/ml BSA) at 65˚C. Following hybridisation, the membranes were washed in four changes of 0.2xSSC/0.5%SDS buffer at 65˚C, before being exposed to BioMax MS-1 autorad film (Kodak). cDNA clones which hybridised strongly to subtracted pistil cDNA, but not to leaf cDNA were identified as pistil-expressed and sequenced by Geneservice (University of Oxford), using the M13 forward universal

primer. Nucleotide sequences were identified using the BLAST algorithm (http://www.ncbi.nlm.nih.-gov/BLAST/), with default parameters. Functional annotation was assigned according to the Gene Ontology (GO) database. The equality of proportions of functional categories was tested by the non-parametric Kruskal Wallis test as implemented in the Statistical Package for the Social Sciences program (SPSS).

Northern blot analysis

Total RNA was extracted from vegetative tissues; root, leaf, stem, capitulum bud, flower bud and pistil tissue using the Plant RNeasy kit (Qiagen) and from pollen using TRI Reagent (Molecular Research Centre Inc.) according to the manufacturer's instructions**.** Total RNA (10µg) from each tissue was separated on a 1.2% agarose formaldehyde gel at 80 V for 4 h and blotted onto Hybond-NX membrane (GE Healthcare) using standard procedures (Sambrook et al., 1989). Following transfer, RNA was immobilized onto the membrane using a CL-1000 Ultraviolet Crosslinker (UVP) set at 120 mJ/cm^2. Probes were prepared using Ready-To-Go™ DNA Labelling Beads (GE Healthcare) and labelled with [α^{-32P}] dCTP (Amersham Biosciences). Northern hybridization was performed at 42˚C, according to Sambrook et al., (1989). After hybridisation, the membranes were washed in four changes of 1xSSC/0.1%SDS buffer at 42˚C, before being exposed to BioMax MS-1 autorad film (Kodak).

Comparison of pistil datasets from different species

Pistil-enriched data sets from *Arabidopsis thaliana* (Swanson et al., 2005; Tung et al., 2005), *Oryza sativa* (Li et al., 2007)*, Crocus sativus* (D'Agostino et al., 2007) and *Nicotiana tabacum* (Quiapim et al., 2009) were used for comparison with the *Senecio* data set. Functional annotation was assigned for genes in each data set according to the Gene Ontology (GO) database. Common genes between data sets were identified using keyword searches. Of these, genes possessing high levels of sequence homology were identified by aligning sequences (see below) and calculating the percentage of sequence identity.

Sequence alignment

DNA and protein sequences were aligned using DNAMAN (Lynnon Corporation). Homology trees were created from multiple alignments using default parameters. The DNAsp package was used to calculate summary statistics of polymorphism data. Phylogenetic trees were generated from alignments using PAUP v 4.0b 10 (Swofford, 2003). Parsimonius trees were generated via a heuristic search with branch swapping set at 1000 rearrangements. Bootstrap calculations were based on 1000 replicates.

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Figure legends

Figure 1. Functional classification of the pistil specific genes from the data sets of A, *Arabidopsis thaliana* (501 genes, Tung et al., 2005; Swanson et al., 2005); B, *Oryza sativa* (115 genes, Li et al., 2007); C, *Senecio squalidus* (115 genes, this study). Functions were assigned according to the Gene Ontology Database. Inset pictures illustrate the structure of the pistil of each species.

Figure 2. Developmental northern blot analysis of candidate stigma specific gene expression. Total RNA was extracted from *S. squalidus* tissues, each lane contains 10 µg total RNA. Lane 1: root, 2: leaf, 3: stem, 4: small capitulum bud (2–3 mm), 5: medium capitulum bud (5–6 mm), 6: large capitulum bud (8-9 mm), 7: open capitulum, 8: floret buds, 9: mix of open florets and buds, 10: florets all open, 11: mature stigmas, 12: pollen. The RNA was probed separately with; A, cytochrome p450 (GO255238); B, calcium binding/kinase (GO255154); C, nodulin (GO255182); D, myo-inositol oxygenase (GO255123); E, Membrane-associated protein (GO255107); F, nematode resistance protein (GO255085); (G represents a loading control).

Figure 3. Sequence alignments and homology trees of conserved pistil-specific proteins from *S. squalidus, O. sativa, A. thaliana, N. tabacum* and *C. sativus*. A, lipid transfer protein, orthologous to SCA *(Lilium longiflorum);* B, Cysteine-rich protein; C, Nodulin/mtn3 family protein, orthologous to mtn3 *(Medicago trunculata)* and NEC1 *(Petunia x hybrida).*

Figure 4. Phylogenetic relationship between Nodulin/mtn3 genes in *A. thaliana* and pistil-specific homologues in *O. sativa* (BAG89875) and *S. squalidus* (GO255182). The parsimonious tree was constructed in PAUP v4.0b 10 using a heuristic search method executed on a protein sequence alignment and rooted with the NEC1 sequence from *Petunia x hybrida* (AAG34696). Bootstrap support values indicated next to nodes, based on 1000 replicates. Pistil-expressed genes are indicated by filled circles; Pollen-expressed genes are indicated by unfilled circles.

Figure S1. The phylogenetic distribution of species most widely used in studies of pollen-pistil interactions. The species for which pistil transcriptome data have been produced are underlined. The stigma state is indicated next to the species. The tree is based on APG II (2003).

Table I. Candidate stigma-specific genes from *Senecio squalidus*.

174 genes were isolated by suppression subtractive hybridisation and assigned a putative function using BLAST. Genes highlighted in bold text represent novel pistil-specific transcripts. ID, identification.

Functional Category	Description	No. cDNAs isolated	Accession number	Arabidopsis ID
Cell wall related				
	Pi-tubulin	3	GO255121	
	Beta-tubulin	1	GO255103	
	Extensin (class I)	10	GO255084	AT1G26250
	Structural molecule	1	GO255092	AT5G54110
	Integral membrane family prot.	1	GO255089	AT4G25040
	Pectinesterase family protein	1	GO255167	AT5G62350
	Alpha-L-arabinofuranosidase	1	GO255144	AT5G49360
	Beta-galactosidase precursor	2	GO255246	AT5G56870
	Beta xylosidase	3	GO255211	AT5G49360
	Proline-rich extensin-like family	1	GO255237	AT5G35190
	Extensin-like protein	1	GO255222	
Stress/defence				
	Stigma-specific peroxidase precursor	5	GO255081	
	Putative nematode-resistance prot.	1	GO255085	AT3G55840
	Nodulin MTN3	1	GO255182	AT4G10850
	Senescence-associated nodulin 1A	3	GO255150	AT3G19000
	Dehydration-responsive protein	2	GO255187	AT3G51070
	EDGP precursor	3	GO255139	AT1G03230
	Aluminum-induced protein	2	GO255210	AT5G43830
	Copper/zinc-superoxide dismutase	2	GO255230	
Hormone-related				
	ABA-inducible protein	3	GO255142	AT5G38760
	Ethylene forming enzyme (EFE)	1	GO255174	
	Auxin/indole-3-acetic acid	1	GO255241	AT3G23050
Metabolism				
	Beta-fructofuranosidase	3	GO255090	AT1G12240
	GDSL-motif lipase/hydrolase family	2	GO255184	AT5G45670
	Mandelonitrile lyase	6	GO255125	AT1G73050
	Galactokinase like protein	2	GO255086	AT4G16130
	Fructose-bisphosphate aldolase	3	GO255113	AT5G03690
	Putative phosphatase	1	GO255111	AT1G17710
	GMC oxidoreductase	3	GO255209	AT1G73050
	Short-chain alcohol dehydrogenase	1	GO255135	AT3G55310
	Cytochrome P450	4	GO255238	AT3G19270
	3-ketoacyl-CoA thiolase	1	GO255148	AT2G33150
	Vacuolar H+ATPase subunit	2	GO255206	
	Serine hydroxymethltransferase	1	GO255188	AT5G61820
	Glycerophosphoryl diester	1	GO255129	AT5G43300
	Hypothetical protein OsJ_006695	1	GO255175	AT5G43300
	2OG-Fe(II) oxygenase	1	GO255176	AT3G11180
	NEC1	1	GO255138	AT5G23660
	Prephenate dehydratase	2	GO255163	AT1G08250

(Table continues on following page.)

Table I. (Continued from previous page).

Functional Category	Description	No. cDNAs isolated	Accession number	Arabidopsis ID
	Xyloglucan endotransglycosylase	1	GO255161	AT4G14130
	UDP-galactose	1	GO255126	AT1G12780
	Lipase, putative	1	GO255164	AT1G28600
	MIOX1 (Myo-Inisitol oxygenase)	1	GO255130	AT2G19800
	Glycosyl hydrolase family 3 protein	1	GO255211	AT5G49360
	Enoyl-CoA hydratase/isomerase	1	GO255232	AT3G60510
	Acetoacetyl CoA thiolase	1	GO255227	AT2G33150
	3-hydroxyisobutyryl-coenzyme A	1	GO255194	AT2G30660
	Glycosyl transferase, family 48	1	GO255243	AT1G05570
Protein fate				
	Putative polyubiquitin	1	GO255110	AT1G65350
	Seven in absentia family protein	1	GO255172	AT4G27880
	Endoplasmatic reticulum retrieval	1	GO255137	AT2G21600
	Structural constituent of ribosome	1	GO255147	AT3G43980
	Binding protein	1	GO255200	AT3G13330
	Cysteine protease	1	GO255232	AT4G31810
	UDP-glucuronate decarboxylase 1	1	GO255100	AT5G59290
	serine carboxypeptidase-like 42	1	GO255215	AT5G42240
Signalling				
	Kinase interacting family protein	1	GO255171	AT1G09720
	GASA-like protein	4	GO255165	AT3G02885
	Shaggy-related protein kinase 1	1	GO255160	AT3G05840
	Protein kinase, MAPK	2	GO255162	AT3G18750
	Calcium-binding protein; kinase	1	GO255154	AT3G48260
	Alpha-kinase	1	GO255168	
	Leucine-rich repeat protein kinase	1	GO255220	AT5G40340
	Somatic embryogenesis receptor kinase	2	GO255098	AT1G71830
	Putative AMP-binding protein	1	GO255112	AT5G16340
	Putative protein kinase; resistance gene	1	GO255082	AT4G35600
	Receptor protein kinase	2	GO255115	AT1G63430
	Protein kinase; Adipokinetic hormone	1	GO255095	AT1G06840
Transcription				
	Transcription factor/ regulator	1	GO255078	AT2G47270
	Leucine rich repeat	1	GO255114	AT5G66650
	Nucleic acid binding	1	GO255153	AT2G02570
	Zinc finger protein	1	GO255216	AT5G04390
	Sunflower 16 protein (SF16)	2	GO255239	AT1G01110
	Serine/threonine protein phosphatase	1	GO255235	AT1G56440
	RNA-binding region RNP-1	2	GO255195	AT2G43370
	Putative reverse transcriptase	6	GO255236	
Transport				
	Iron transporter protein IRT1	1	GO255096	AT4G19690
	Putative auxin efflux carrier protein 9	1	GO255104	AT1G73590
	Heavy metal transport detox protein	2	GO255118	AT1G12520
	FAD-binding domain-containing prot.	1	GO255105	AT4G20830

(Table continues on following page.)

Table I. (Continued from previous page).

Functional Category	Description	No. cDNAs isolated	Accession number	Arabidopsis ID
	Stigma/style ABC transporter	2	GO255101	AT5G13580
	Membrane associated protein	1	GO255107	AT5G54110
	Calcium-binding EF hand family protein	2	GO255128	AT2G34030
	E-class P450, group I	2	GO255238	AT3G26330
	Lipid-transfer protein	2	GO255151	AT5G59310
	Amino acid carrier	1	GO255185	AT5G09220
	PIP1 aquaporin	1	GO255146	AT4G00430
	Endoplasmic reticulum ATPase	1	GO255244	AT5G03340
	Cytochrome oxidase subunit I	1	GO255196	AT3G57450
	Cysteine proteinase	1	GO255193	
	FAD linked oxidase, N-terminal	1	GO255221	AT3G63440
	Ras-related GTP-binding protein	1	GO255224	AT1G02130
	Zinc ion binding	1	GO255248	AT3G60520
Unclassified				
	Sunflower 21 protein (SF21)	2	GQ227732	AT2G19620
	Putative proline-rich protein	1	GO255157	AT5G45670
	Hypothetical protein	1	GO255217	
	Unknown protein	2	GO255213	AT3G46070
	Cytokine induced apoptosis inhibitor	1	GO255116	AT5G18400
	Cupin family protein	1	GO255124	
	Hypothetical protein	1	GO255119	
	Uncharacterized Cys-rich domain	1	GO255145	AT2G37110
	Unknown protein	1	GO255192	AT4G35710
	Expressed protein [Oryza sativa]	1	GO255156	
	Hypothetical protein	1	GO255178	AT5G06380
	LIM domain protein PLIM-2	1	GO255173	AT1G01780
	Gag polyprotein	1	GO255127	
	CBS domain-containing protein	1	GO255201	AT3G48530
	Sunflower 3 protein (SF3)	1	GO255242	AT1G10200
	Unknown protein	2	GO255233	AT1G54320
	Hypothetical protein	1	GO255205	AT3G22850
	GEG protein	1	GO255228	AT2G18420
	Unknown protein	1	GO255207	AT3G57450
	Hypothetical protein	1	GO255247	AT3G52710
	DENN domain containing protein	1	GO255219	AT5G35560
	Hypothetical protein	1	GO255189	AT2G19800

Table II. Senecio squalidus *pistil-expressed genes which have been identified in at least two of the pistil preferential datasets from different species. The study species used were:* Arabidopsis thaliana *(Tung et al., 2005; Swanson et al., 2005),* Oryza sativa *(Li et al., 2007),* Nicotiana tabacum *(Quiapim et al., 2009) and* Crocus sativus *(D'Agostino et al., 2007).* *Presence/absence is indicated for each species, followed by transcript numbers in brackets.*

Senecio squalidus 	*Arabidopsis thaliana* 	*Oryza sativa* 	*Crocus sativus* 	*Nicotiana tabacum* 
Cytochrome p450	✓ (6)	✓ (8)	✓ (6)	✓ (1)
Lipid transfer protein	✓ (4)	✓ (1)	✓ (2)	✓ (1)
3-ketoacyl-coA synthase	×	✓ (1)	✓ (1)	×
Acyl-CoA-binding protein	✓ (1)	✓ (1)	✓ (1)	×
Beta-xylosidase	×	✓ (3)	✓ (1)	×
Beta-galactosidase prec.	×	✓ (3)	✓ (1)	×
UDP-glycosyltransferase	✓ (4)	✓ (1)	✓ (5)	×
Zinc finger protein	×	✓ (2)	✓ (6)	✓ (1)
Peroxidase	✓ (3)	✓ (3)	✓ (1)	×
Aquaporin	✓ (1)	✓ (1)	✓ (1)	×
FAD oxidoreductase	✓ (1)	×	✓ (1)	×
ABC transporter family	✓ (1)	✓ (3)	✓ (2)	✓ (3)
Extensin-like protein	✓ (1)	×	✓ (1)	✓ (7)
Cysteine protease	✓ (1)	✓ (1)	✓ (4)	×
Mandelonitrile lyase	✓ (2)	×	×	×
Pectinesterase	✓ (2)	✓ (3)	✓ (1)	×
Serine carboxypeptidase	✓ (2)	✓ (1)	×	×
Receptor protein kinase	✓ (2)	✓ (5)	✓ (4)	✓ (4)
Disease resistance	✓ (1)	✓ (5)	✓ (1)	✓ (1)
Lipase/hydrolase	✓ (1)	✓ (1)	×	×
Cysteine-rich protein	✓ (1)	✓ (1)	×	×
Nodulin/mtn3	✓ (3)	✓ (2)	×	✓ (2)

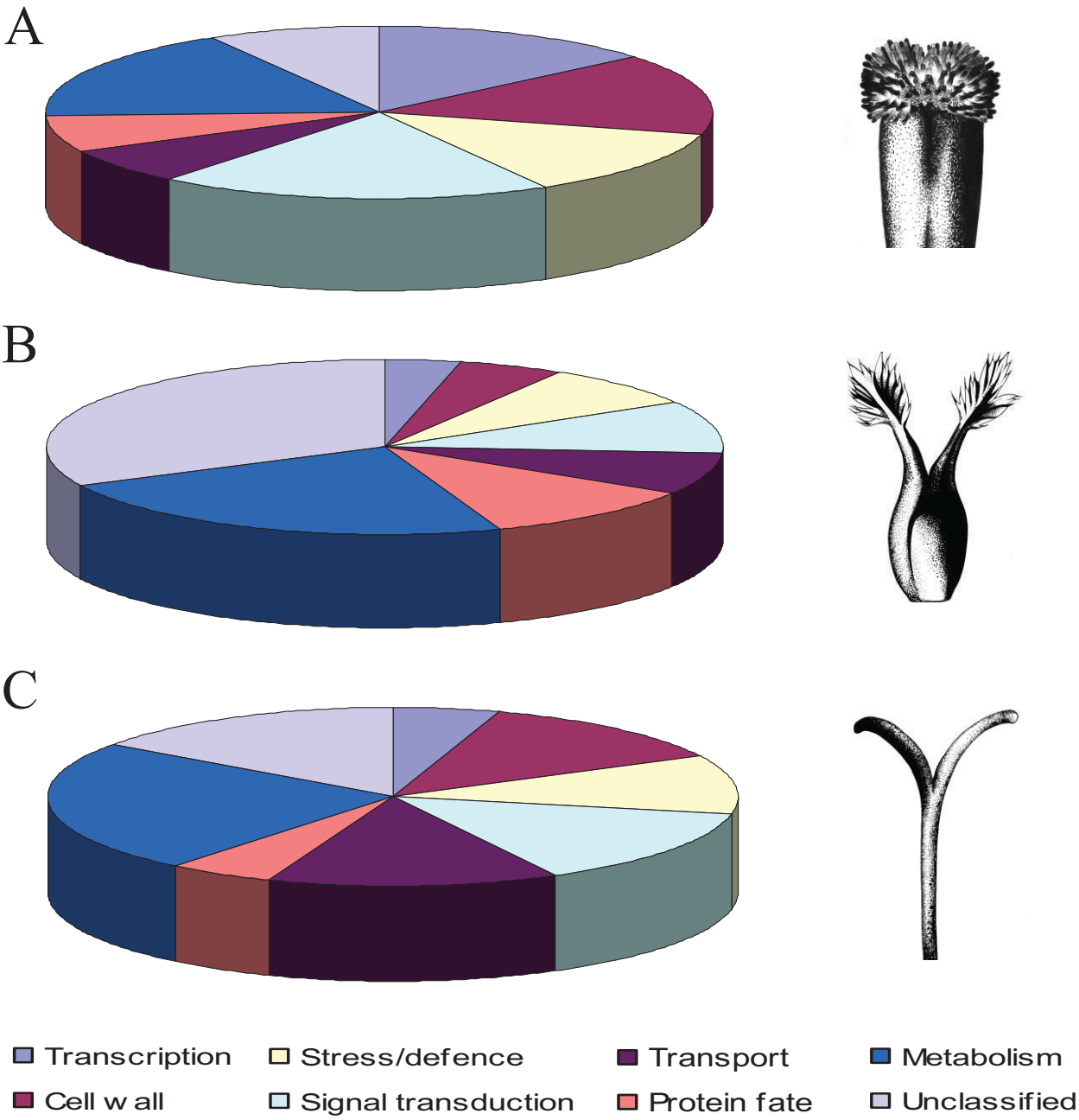

Figure 1. Functional classification of the pistil specific genes from the data sets of A, *Arabidopsis thaliana* (501 genes, Tung et al., 2005; Swanson et al., 2005); B, *Oryza sativa* (115 genes, Li et al., 2007); C, *Senecio squalidus* (114 genes, this study). Functions were assigned according to the Gene Ontology Database. Inset pictures illustrate the structure of the pistil of each species.

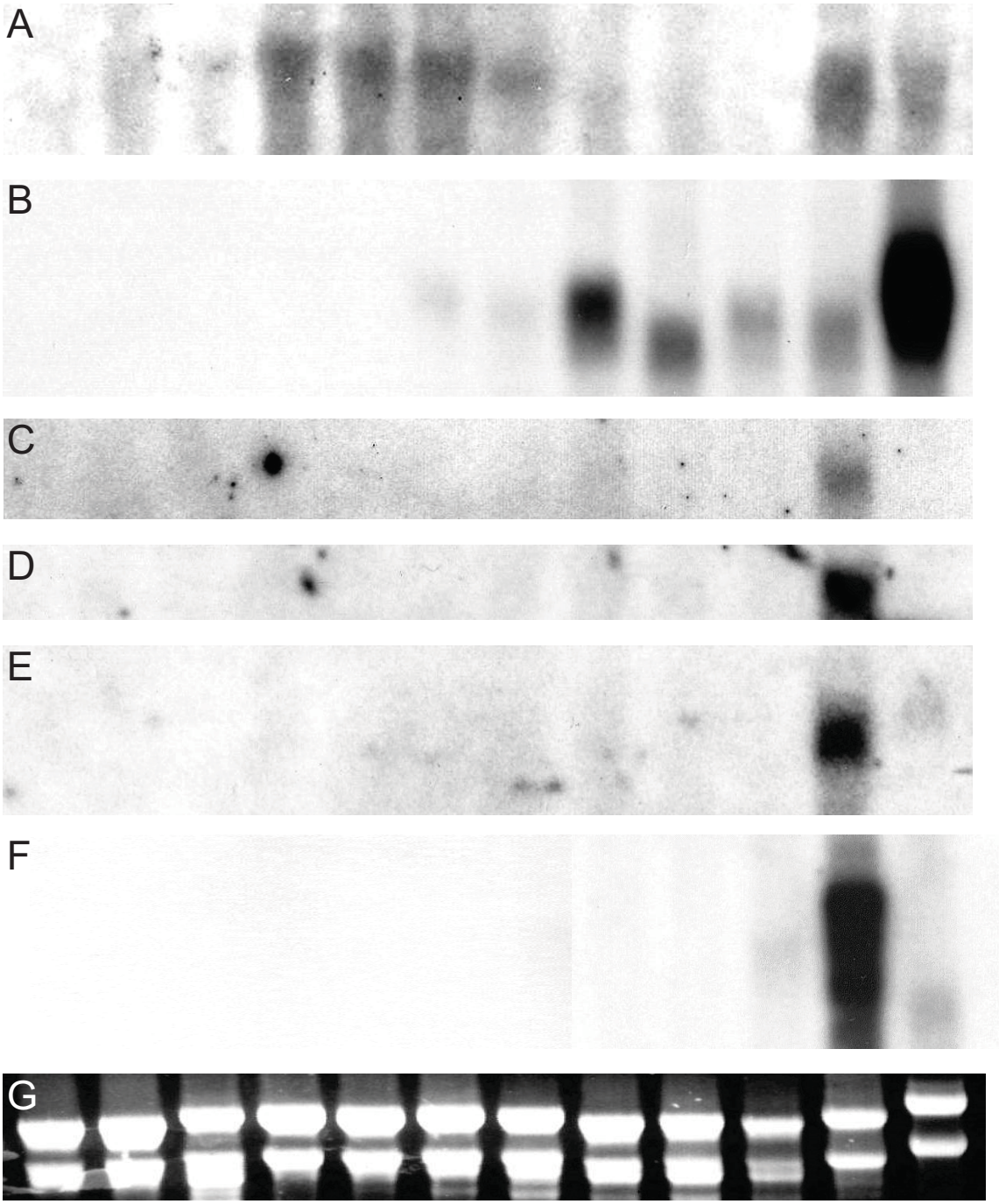

Figure 2. Developmental northern blot analysis of candidate stigma specific gene expression. Total RNA was extracted from *S. squalidus* tissues, each lane contains 10 µg total RNA. Lane 1: root, 2: leaf, 3: stem, 4: small capitulum bud (2–3 mm), 5: medium capitulum bud (5–6 mm), 6: large capitulum bud (8-9 mm), 7: open capitulum, 8: floret buds, 9: mix of open florets and buds, 10: florets all open, 11: mature stigmas, 12: pollen. The RNA was probed separately with; A, cytochrome p450 (GO255238); B, calcium binding/kinase (GO255154); C, nodulin (GO255182); D, myo-inositol oxygenase (GO255123); E, nematode resistance protein (GO255085); F, Membrane-associated protein (GO255107) (G represents a loading control).

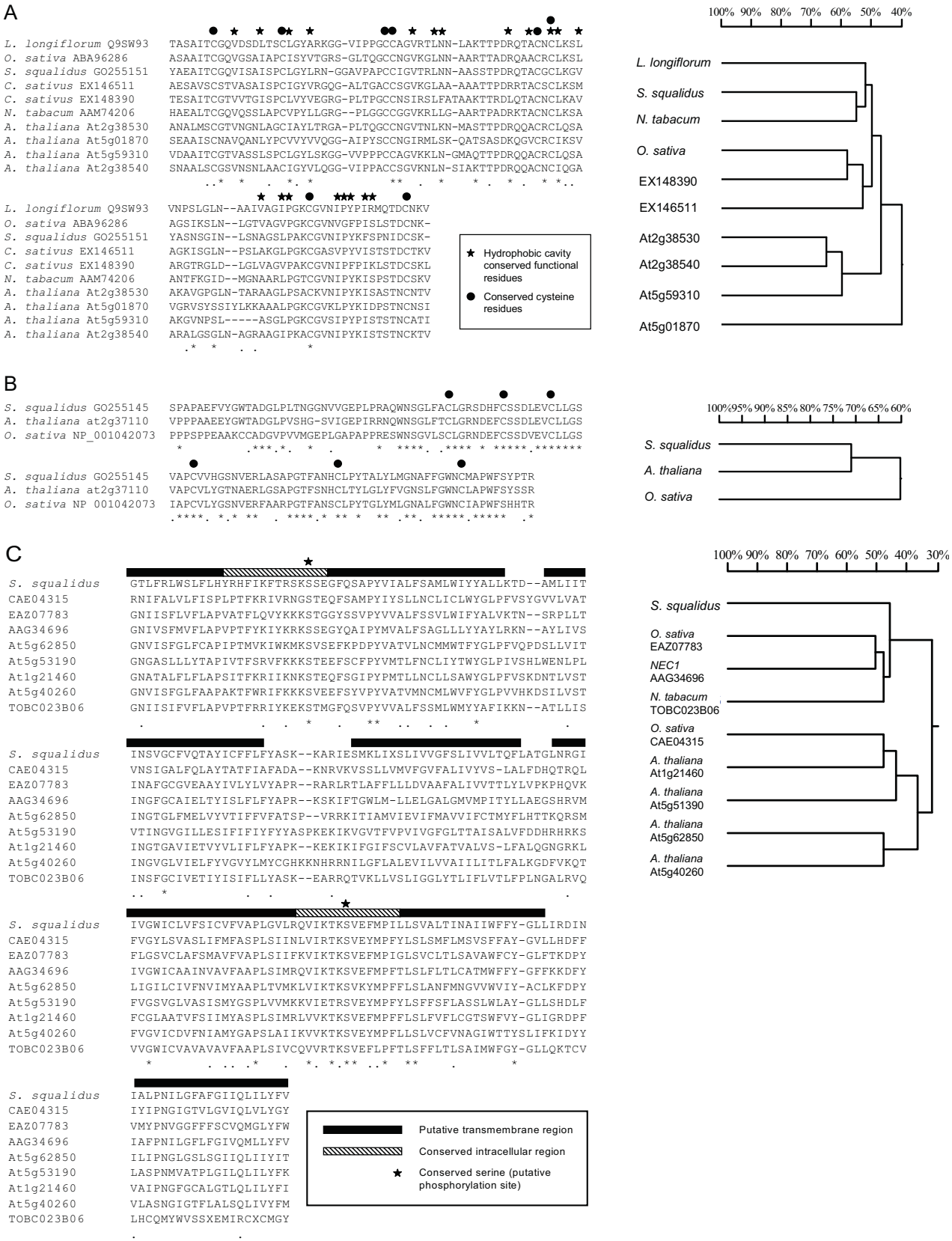

Figure 3. Sequence alignments and homology trees of conserved pistil-specific proteins from *S. squalidus, O. sativa, A. thaliana, N. tabacum* and *C. sativus*. A, lipid transfer protein, orthologous to SCA *(Lilium longiflorum);* B, Cysteine-rich protein; C, Nodulin/mtn3 family protein, orthologous to mtn3 *(Medicago trunculata)* and NEC1 *(Petunia x hybrida).*

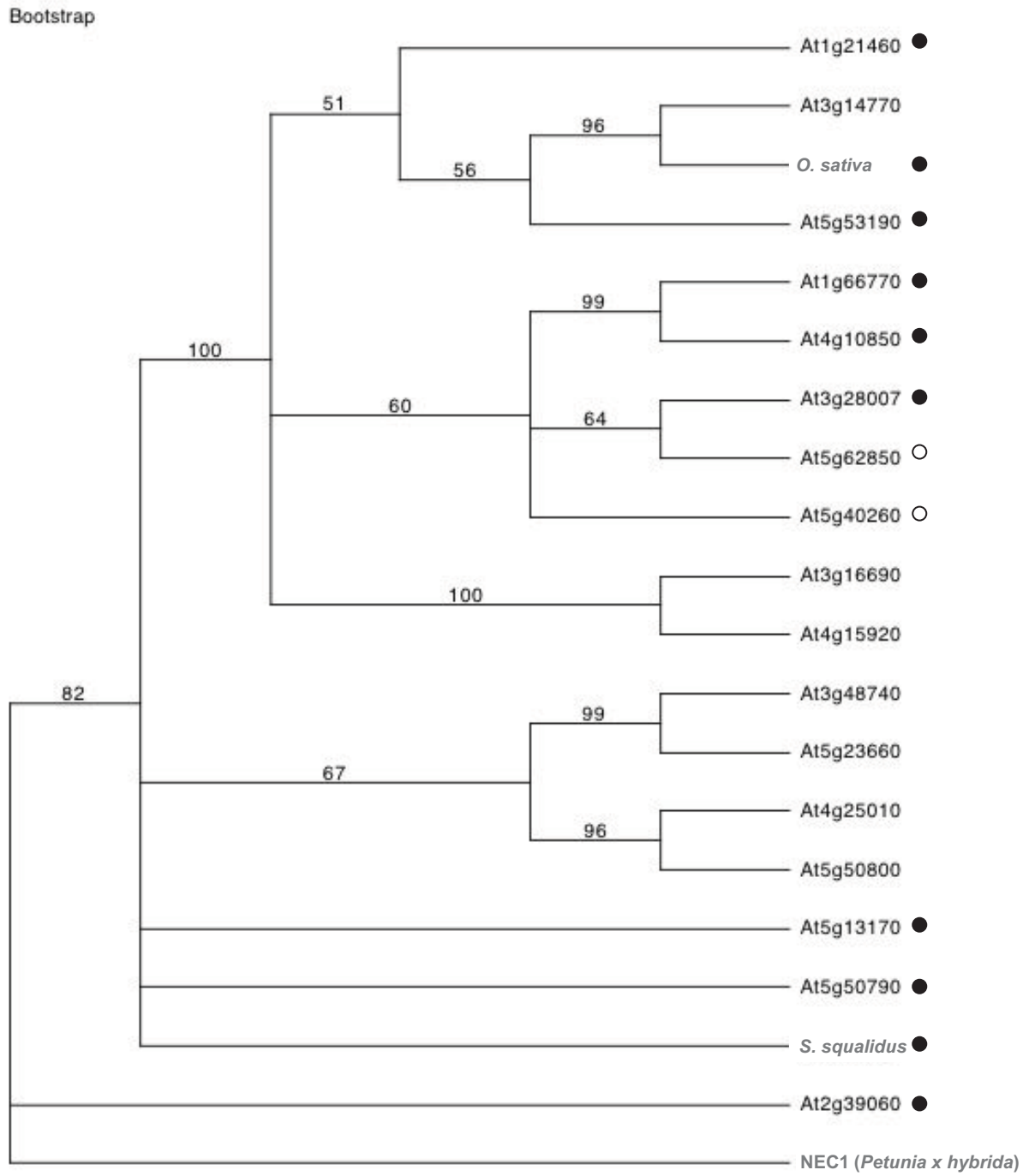

Figure 4. Phylogenetic relationship between Nodulin/mtn3 genes in *A. thaliana* and pistil-specific homologues in *O. sativa* (BAG89875) and *S. squalidus* (GO255182). The parsimonious tree was constructed in PAUP v4.0b 10 using a heuristic search method executed on a protein sequence alignment and rooted with the NEC1 sequence from *Petunia x hybrida* (AAG34696). Bootstrap support values indicated next to nodes, based on 1000 replicates. Pistil-expressed genes are indicated by filled circles; Pollen-expressed genes are indicated by unfilled circles.